Possible involvement of *put A* gene in *Helicobacter pylori* colonization in the stomach and motility

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ABSTRACT

H. pylori is a gram-negative bacterium associated with gastric inflammation and peptic ulcer and considered a risk factor for gastric cancer in its natural habitat. However, the energy metabolism of *H. pylori* in the stomach remains to be clarified. *H. pylori* shows rather high respiratory activity with L-proline and significantly large amounts of L-proline are present in the gastric juice from *H. pylori* infected patients. We constructed a disrupted mutant of the *put A* gene, which encodes the proline utilization A (Put A) flavin-linked enzyme, in order to examine the role of *put A* in the gastric colonization of *H. pylori*. The *put A* disrupted mutant, $\Delta putA$, was constructed by inserting a chloramphenicol resistant gene into *put A*. $\Delta putA$ did not show respiratory activity using L-proline and could not incorporate L-proline into cells. $\Delta putA$ also did not show motility in response to amino acids and did not display the swarming activity observed with the wild-type. $\Delta putA$ had lost its ability to colonize the stomach of nude mice, an ability possessed by the wild-type. These findings indicate that *put A* may play an important role in *H. pylori* colonization on the gastric mucus layer.

Helicobacter pylori is a gram-negative bacterium associated with gastric inflammation and peptic ulcer and is a risk factor for gastric cancer (4, 8, 27, 32). The natural habitat of *H. pylori* is the mucus layer on the human gastric epithelium, where it is considered to survive for more than ten years. This micro-aerophilic bacterium does not catabolize saccharides (19), and the preferred energy substrate is not glucose (20, 21) but organic acids and amino acids.

However, the energy metabolism of *H. pylori* in its natural habitat has not been clarified. We previously reported that *H. pylori* shows rather strong respiratory activity when amino acids such as L-proline, L-serine and L-alanine are used as respiratory substrates (22). We also reported that the gastric juice of patients infected with *H. pylori* contained rather large amounts of these amino acids, especially L-proline (22). Thus, we proposed that *H. pylori* may utilize these amino acids as energy sources in the stomach.

Recently, Guszczyn *et al.* reported that a free-proline pool is present in human gastric tissue, where there are extremely large amounts of proline, 10 mg per g of gastric tissue (9). Since the gastric tissue contains much prolidase, this proline seemed to

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have been driven out from the collagen in gastric tissues (9). Kavermann *et al.* demonstrated that *H. pylori* cells secreted collagenase which is encoded by a gene, *hp0169* of *H. pylori* (14). They also described collagenase as being essential for gastric colonization of *H. pylori* (14). In addition, the growth of *H. pylori* was reported to be enhanced by addition of L-proline together with L-alanine to Dulbecco's modified Eagle's minimal essential medium (33). These reports suggest that there are large amounts of proline in the deeper part of the gastric mucus layer, the primary habitat of *H. pylori*. This offers support for our proposal that *H. pylori* cells predominantly utilize L-proline as an energy source (8).

Proline is converted to glutamate in two successive steps by the proline utilizing A (Put A) flavin-linked enzyme in gram-negative bacteria. Put A contains a proline dehydrogenase (EC 1.5.99.8) domain that catalyzes the flavin adenine dinucleotide-dependent oxidation of proline to Δ^1 -pyrroline-5-carboxylate (P5C) and a P5C dehydrogenase (EC 1.5.1.12) domain that catalyzes the NAD⁺-dependent oxidation of P5C to glutamate. The gene *put A* encodes both proline dehydrogenase and P5C dehydrogenase in many gram-negative bacteria including *H. pylori* (3, 10, 15–17, 30, 34). In the present work, we constructed a disrupted mutant of *put A* and investigated the role of *put A* in *H. pylori* colonization in the stomach of the nude mouse. The *put A* disrupted mutant, Δ *put A*, was constructed from two strains of *H. pylori*, ATCC 43504 and HPK-5 by insertion of a chloramphenicol resistant gene into the middle of *put A*. The results showed that the Δ *put A* mutant from the two strains did not display respiratory activity of L-proline because the mutant had lost the activity of proline dehydrogenase which is essential for proline respiration. *H. pylori* displays rather high respiratory activity with L-proline, and hydrogen derived from L-proline by L-proline dehydrogenase probably participates in ATP production via the respiratory chain of *H. pylori* as described previously (22). Interestingly, the Δ *put A* from both strains had little motility and did not show swarming activity. In addition, no colonization occurred in the stomach of nude mice infected with this mutant. The motility of *H. pylori* is one of the important virulent factors, as no colonization in the stomach is observed in mice infected with a strain with disrupted flagella (6). Thus, the failure of Δ *put A* to cause colonization may be due to its loss of motility.

This is the first report to suggest that the *put A* gene related to L-proline respiration may be essen-

tial for the motility necessary for *H. pylori* to colonize the gastric mucus layer.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of *H. pylori* used in the present study, ATCC 43504 and HPK-5 (31), were cultured in Brucella broth (Becton Dickinson, Cockeysville, MD, USA) containing 5% horse serum (Donor Horse Serum manufactured by Thermo Trace Ltd., Melbourne, Australia) under 10% CO₂ in an incubator with gentle shaking at 37°C for 20 h as described previously. *Escherichia coli* JM109 was used for the sub-cloning of *put A*. *E. coli* was cultured in Luria-Bertani broth or agar medium supplemented with kanamycin (50 µg/mL) or chloramphenicol (30 µg/mL) at 37°C.

DNA techniques. DNA and plasmid purification, and cloning procedures were performed as described previously (5). *H. pylori* genomic DNA was extracted with a Genomic DNA Extraction Miniprep System (VIOGENE, Sunnyvale, CA, USA). Plasmid DNA was prepared and purified by using a Miniprep plasmid extraction kit (Bio Rad, CA, USA). PCR was performed with a TaKaRa PCR Thermal Cycler MP (TAKARA BIOINC., Shiga, Japan). Oligonucleotide primers were synthesized by Texas Genomics, Japan.

Construction of Δ *put A* mutant of *H. pylori*. Primers 56F1 (5'-ATGCAAAAAATCATTGACGATTTCGCTAG-3') and 56R1 (5'-TTTTCAGCACAGCATGATTGTGGCAAG-3') were designed based on the *H. pylori* 26695 strain and used for cloning of *put A*. The Xho I restriction site of pCRXL cloning vector (Invitrogen, Carlsbad, CA, USA) was deleted by inserting a DNA fragment (5'-TCGACGTCGA-3'). Next, the Xho I-site was inserted into the middle of *put A*, and pCRXL:*put A* was amplified by PCR with the primer pair of 56XHOF (5'-GAGCTTTACCCAGAGATGGATGGGAGGTTTGAA GAT-3') and 56XHOR (5'-ATCCATCTCTGGGTA AAGCTCGAGTTAAAATAGGAGCGTTACG CAT-3'). The green fluorescent protein:chloramphenicol (GFP. Cm) cassette was inserted into the Xho I site. The recombinant plasmid was then introduced into *H. pylori* by natural transformation via allelic exchange, and chloramphenicol-resistant colonies were isolated. These genomic mutants were confirmed by Southern blotting methods and DNA sequencing.

Fig. 1 DNA constructions. (a) Schema of *put A* (HP0055) and *put P* (HP0056) genes locations in wild type, 26695 strain. (b) *Put A* was amplified by PCR and inserted into pCRXL cloning vector. (c) *GFP:Cm* cassette was inserted into the middle of the amplified *put A* gene of pCRXL:*put A* vector. (d) Isogenic *put A* disrupted mutants of ATCC43504 and HPK5 were obtained by allelic exchanged mutagenesis with pCRXL:*put A*:*GFP* vector.

Assay of 2,6-Dichlorophenolindophenol (DCIP) reducing and respiratory activities by *H. pylori* whole cells. *H. pylori* cells were added to the reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.01 M sodium azide, 10 mM L-proline and 50 μ M DCIP. After 10 min incubation at 37°C, the cells were removed by centrifugation at $10,000 \times g$ for 5 min and then A₆₀₀ of the supernatant was measured using DU 640, Beckman, Fullerton, USA. An ϵ_{600} of $21.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used as described previously (22).

Cultured *H. pylori* cells were harvested by centrifugation at $3,500 \times g$ for 10 min at room temperature, and suspended in 10 mM Hepes buffer (pH 7.0) containing 0.9% NaCl. Respiration of whole cells (1×10^8 cells/mL) was monitored polarographically using a Clark-type oxygen electrode (YSI Inc., Yellow Springs, OH, USA) in a semiclosed vessel containing the same buffer as above at 37°C as described previously (22). Various substrates were added to the oxygen electrode vessel with a syringe. Respiratory activity (oxygen uptake) was determined from polarographic traces of the oxygen electrode as described previously (22).

Incorporation of L-proline into wild-type and Δ *put A* cells of *H. pylori*. Cultured *H. pylori* cells from wild-type and Δ *put A* of ATCC 43504 were harvested by centrifugation as described above. Cells (1×10^8) were suspended in one mL of Ham's F-12 medium prepared according to Gibco Catalog No. 21700 with bicarbonate. Five bottles of 20 mL tissue culture bottles, each containing 5 mL of the cell suspension, were cultured microaerobically at 37°C with gently shaking. Cells were harvested at 0, 5, 10, 24 and 30 h, then centrifuged at $3,500 \times g$ for 10 min at room temperature to obtain the cell-free supernatant. The amount of L-proline in the supernatant was assayed as described previously (22).

Determination of free-amino acids in wild-type and Δ *put A* cells of *H. pylori*. Wild-type and Δ *put A* cells from *H. pylori* ATCC 43504 were cultured in Brucella broth containing 5% of horse serum as described above. After 20 h cultivation, cells were harvested by centrifugation. The amounts of free-amino acids present within cells of both the wild-type and Δ *put A* strains were assayed as described previously (22).

Assay of the motility responding to amino acids in H. pylori whole cells. *H. pylori* cells grown microaerobically were collected and washed twice with 50 mM Na-K phosphate buffer (pH 7.0) containing 0.1 mM MgSO₄. Various kinds of amino acids (1 mM, final concentration) were added to the same buffer described above containing whole cells (1×10^7 cells/mL). A total of 80 μ L of the cells was placed into the chamber covered with a glass slip. The response of each bacterial strain to the amino acids on the thermoplate (MATS-555F, Olympus, Japan) was observed using a microscope.

Assessment of swarming activity. The swarming in a soft-agar plate containing Brucella broth, 5% horse serum, 0.3% agar and 0.8% gelatin was assayed. *H. pylori* ATCC 43504 cells cultured with the Brucella broth described above were centrifuged, and the resulting pellet was pressed onto the soft agar plate. After 3–4 days of cultivation as described above, the swarming was assessed from the diameter size of the colony.

Electron microscopy. The morphology of flagella from wild-type and Δ put A *H. pylori* ATCC 43504 was examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *H. pylori* strains were cultured with Brucella broth as described above. For SEM, samples were prepared by fixing *H. pylori* cells in 1% glutaraldehyde, and washed with 0.1 M sodium cacodylate buffer (pH 7.2) over-night at 4°C. The samples were then dehydrated using a graded acetone series (30, 50, 70, 95 and 100%). Subsequently, the samples were dried with a critical point dryer (HCP-1; Hitachi, Ibaragi, Japan) and sputter-coated with gold palladium (E-5150; Polaron, Watford, U.K.). Images of the cells were obtained with SEM (S-800; Hitachi, Tokyo, Japan). For TEM, the cells were negatively stained as described previously (11). The cells were then mounted on polyvinyl formvar membrane and negatively stained with 2% uranyl acetate. Images of the cells were scanned by an electron microscope (H-7100; Hitachi, Tokyo, Japan).

Infection of nude mice with wild-type and Δ put A. Female Balb/c nude mice aged 6 weeks (Japan SLC, Inc., Shizuoka, Japan) were used. Wild-type and Δ put A cells from ATCC 43504 or HPK-5 were orally administered with 1×10^8 cells to mice after 12 h of fasting, twice with a three-day interval. Five mice of each strain were infected, and the stomachs were obtained after 4 weeks. Colony forming unit

(CFU) tests were carried out to assess the colonization in the stomachs of the mice as described previously (29).

Determination of protein. The amount of protein in *H. pylori* cells was determined by a modification of the Lowry procedure (18).

RESULTS

DCIP reducing and respiratory activities in wild-type and Δ put A cells from H. pylori

Δ put A mutant from ATCC 43504 and HPK-5 completely lacked the activity of L-proline dehydrogenase which reduces DCIP using hydrogen from L-proline as shown in Table 1. *H. pylori* has a gene encoding D-amino acid dehydrogenase (*dad A*), which reduces also DCIP using hydrogen from D-proline or D-alanine. The Δ put A from these two strains had DCIP-reducing activities of D-proline and D-alanine at almost the same levels as those of their wild strains (Table 1).

Respiratory activity by L-proline depends on L-proline dehydrogenase in *H. pylori*. The Δ put A from these two strains did not display respiration by L-proline as shown in Table 2, while respiratory activities of other amino acids, such as L and D isomers of alanine and L-serine did not show any significant difference between the Δ put A and the wild-type of ATCC 43504 and HPK-5.

Utilization of proline in wild-type and Δ put A of H. pylori

H. pylori has a gene of *put P* which encodes a proline permease responsible for the incorporation of proline into whole cells. Since *put P* is situated downstream of *put A*, the disruption of *put A* may affect the transcription of *put P*. We examined proline-incorporation into cells in both the Δ put A and the wild-type of ATCC 43504 by using Ham's F-12 which is a medium containing defined ingredients. With the wild-type, the amounts of proline in the medium decreased time-dependently during incubation at 37°C, and proline almost disappeared after 24 h incubation as shown in Fig. 2. However, the amounts of proline in the medium did not change in the case of Δ put A (Fig. 2). These results suggest that Δ put A seems to have lost the transcribing activity possessed by *put P*.

Proline is not an essential amino acid for the growth of *H. pylori* (28), indicating that *H. pylori* has an enzyme system involved in the *de novo* synthesis of proline. In fact, Δ put A cells contain a

Table 1 DCIP reduction* by wild-type and $\Delta put A$ of *H. pylori* cells with D- and L- isomers of proline and alanine as substrates

Substrate	ATCC43504		HPK-5	
	wild-type	$\Delta put A$	wild-type	$\Delta put A$
L-Proline	7.41	0.00	6.90	0.00
D-Proline	6.30	5.75	6.40	5.35
D-Alanine	5.46	6.42	7.21	7.01

*DCIP reduction : nmol DCIP reduced / min / mg protein

Table 2 Respiration* by wild-type and $\Delta put A$ of *H. pylori* cells with D- and L- isomers of alanine, proline, and serine as substrates

Substrate	ATCC43504		HPK-5	
	wild-type	$\Delta put A$	wild-type	$\Delta put A$
L-Alanine	29.8	34.5	31.7	36.3
D-Alanine	12.8	14.1	24.1	23.0
L-Proline	36.6	0.0	32.2	0.0
D-Proline	27.2	18.9	28.1	21.7
L-Serine	43.4	48.6	57.2	46.0
D-Serine	8.9	14.1	13.3	15.4

*Respiration: nmol oxygen / min / mg protein

Fig. 2 Incorporation of L-proline into wild-type and $\Delta put A$ of *H. pylori* ATCC 43504. Closed circles with solid line and open circles with dotted line show wild-type and $\Delta put A$, respectively. Experimental protocol is described in MATERIALS AND METHODS. Percentage of control was determined using the following equation: percentage = (amounts of L-proline in Ham's medium F-12) / (amounts of L-proline in cell-free supernatant of culture medium after 0, 5, 10, 24 and 30 h)⁻¹(100)

large amount of proline (Fig. 3), suggesting that *put A* disruption does not seem to affect on the biosynthesis of proline. While the wild-type cells had only a small amount of proline, their amounts of glutamate were significantly higher than those in $\Delta put A$ cells (Fig. 3). These results suggested that wild-type

cells dominantly metabolized L-proline into glutamate with their dehydrogenase. Unlike the cases of proline and glutamate, the amounts of other amino acids such as alanine, serine and aspartate did not show significant difference between the wild-type and the $\Delta put A$ strains (Fig. 3). These results offer support for proline being dominantly used as a respiratory substrate in the wild-type *H. pylori*, but not in $\Delta put A$.

Motility responding to amino acids in wild-type and $\Delta put A$ of *H. pylori*

H. pylori ATCC 43504 cells showed that their motility responded to amino acids depending on their type as can be seen from Table 3. *H. pylori* cells showed motile activity responding to alanine, proline, serine, and asparagine. Among these amino acids, *H. pylori* showed the strongest motile activity with proline. However, $\Delta put A$ showed no motility with respect to proline, and only a little motility in response to other amino acids (Table 3). Although the data are not shown, almost the same results as those for the ATCC 43504 strain were obtained for HPK-5.

As *H. pylori* displays swarming activity, we examined this activity of $\Delta put A$ from ATCC 43504 on the plate containing Brucella soft agar. The $\Delta put A$ formed a much smaller zone of swarming, unlike the case of the wild-type (Fig. 4).

Fig. 3 Amount of free amino acids in cells of wild-type and $\Delta put A$ of *H. pylori* ATCC 43504. Closed and open bars show wild-type and $\Delta put A$, respectively. Experimental protocol is described in MATERIALS AND METHODS.

Table 3 Motility responding to various amino acids in wild-type and $\Delta put A$ of *H. pylori* ATCC43504 cells

Amino acid	<i>H. pylori</i> ATCC43504	
	wild-type	$\Delta put A$
Alanine	+++	±
Serine	+++	±
Proline	+++++	–
Asparagine	+++	±

Cells under a microscope were showing non motile activity (–), and < 10% (±), about 60% (+++) and > 90% (+++++) of cells under a microscope were showing motile activity.

Electron microscopic observation of flagella of wild-type and $\Delta put A$

Since the motility of *H. pylori* depends on the presence of flagella, we compared the morphology of the flagella between the wild-type and the $\Delta put A$ from ATCC 43504 by electron microscopy. As shown in Fig. 5, the flagella of $\Delta put A$ seem to exist (d), but full-length sheathed flagella like those of the wild-type (b, c) were rarely observed. Instead, the flagella of $\Delta put A$ were short in length and seemed to be deformed (e, f), therefore, they were very difficult to identify as flagella.

ATCC43504

Fig. 4 Swarming abilities of wild-type and $\Delta put A$ of *H. pylori* ATCC 43504. Experimental protocol is described in MATERIALS AND METHODS.

Colonization of wild-type and $\Delta put A$ in the stomach of nude mice infected with *H. pylori*

Nude mice have been used as an experimental model for *H. pylori* colonization in stomach infection (13). To examine whether $\Delta put A$ can or can not display colonization with this model, the animals were infected with either wild-type or $\Delta put A$ of ATCC 43504. The CFU test was used to estimate the colonization in the stomach after 4 weeks of infection. As shown in Fig. 6, positive CFU in four of

Fig. 5 Flagella of wild-type (a-c) and $\Delta put A$ (d-f) of *H. pylori* ATCC 43504 by scanning (SEM) and transmission electron microscope (TEM). a and d: SEM images. b, c, e and f: TEM images after negative staining. Bars show 2 μ m.

Fig. 6 Colonization of wild-type and $\Delta put A$ of *H. pylori* ATCC 43504 to nude mice. Five mice per group were infected either with wild-type or $\Delta put A$ of the *H. pylori*. Each point shows CFU per stomach of a mouse. Other experimental protocols are described in MATERIALS AND METHODS.

five mice was observed in the case of the wild-type from ATCC 43504, but not at all for the $\Delta put A$ mutant. Although data are not shown, the stomach from mice infected with wild-type as well as $\Delta put A$ of HPK-5 did not show positive CFU.

DISCUSSION

Several potential factors including urease, cag A, flagella and chemotactic factors have been reported to be involved in the colonization of *H. pylori* in the stomach of animals (6, 7, 24). However, little work has been done on the colonization of *H. pylori* related to energy metabolism using disrupted mutant strains. Olson and Maier reported recently that *H. pylori* cells used molecular hydrogen as an energy source in mice (26). In their study, the hydrogenase-disrupted mutant did not display colonization in the stomach of mice, where there are many kinds of anaerobic bacteria present at a high density and the molecular hydrogen derived from these anaerobic bacteria seems to be abundant.

In humans unlike in mice, there are few bacteria in the stomach. Since the habitat of *H. pylori* is the gastric mucus layer, various amino acids diffusing from the stomach juice may be utilized by *H. pylori* as an energy source. In our previous report, we showed that human stomach juice contains significant amounts of amino acids such as proline, alanine and serine that can be utilized by *H. pylori* as respiratory substrates (22). We also reported a significantly high content of L-proline in the stomach juice from *H. pylori* infected patients. This led us to propose that *H. pylori* may predominantly use L-proline for an energy source in the human stom-

ach. Support for our hypothesis comes from recent reports. For example, human stomach tissue contains extremely high amounts of free-proline which seems to be derived from collagen in the tissue (9). Also, *H. pylori* cells secrete their own collagenase outside cells, and this enzyme is essential for the colonization in the *H. pylori* infected mouse (14). Collagen, which is present in the extracellular matrix of the stomach, is probably degraded by collagenase and prolidase, resulting in the production of free-proline in stomach tissue. *H. pylori* cells adhere near the tight junctions of the gastric epithelial cells (11, 25). This specific location on the epithelium is thought to be favored by *H. pylori* due to the proximity of bacterial nutrients released by the host tissue. Thus, a niche for *H. pylori* could be expected to contain large amounts of L-proline which has diffused from the stomach tissue. As a result, *H. pylori* cells may utilize L-proline predominantly as a respiratory substrate for an energy source.

In the present work, we have shown that a gene, *put A*, may play an important role in the colonization of *H. pylori*. *Put A* encodes two enzymes, proline dehydrogenase and P5C dehydrogenase. *Put A* was disrupted by insertion of a Cm resistant gene into its middle section using plasmid pCR-XL (Fig. 1). The disrupted mutant of *put A* from two kinds of *H. pylori* strains, ATCC 43504 and HPK-5 did not show respiratory activities of L-proline as well as L-proline dehydrogenase which reduces DCIP using L-proline (Table 1). The $\Delta put A$ mutant from ATCC 43504 did not colonize the stomach of nude mice infected with these strains, although its wild strain could (Fig. 6). The loss of this ability by $\Delta put A$ seems to be due to the following two reasons: (1) the $\Delta put A$ from the two strains lost respiratory activity with L-proline because of the lack of L-proline dehydrogenase activity (Tables 1 and 2) and (2) cells of $\Delta put A$ were nonmotile, showing no swarming activity (Fig. 4).

Motility has been shown to be a key factor in the ability of *H. pylori* to colonize the gastric mucosa (7, 14). Because the gastric mucous gel layer has a rapid turnover, *H. pylori* cells proliferating in the mucous layer need the ability to move toward the epithelial cell surface against the mucous flow toward the duodenum. We have shown here that *H. pylori* exhibited motility responding to various kinds of amino acids. Among the amino acids utilized by *H. pylori* as respiratory substrates, the wild-type strain of ATCC 43504 and HPK-5 showed strong motility responding to proline (Table 3). However, the $\Delta put A$ of these strains had completely lost their motility

responding to proline, and showed little motility in response to other amino acids (Table 3). As a result, $\Delta put A$ from ATCC 43504 had lost its swarming activity (Fig. 4). The flagella of $\Delta put A$ seemed to be deformed when compared with those of the wild-type (Fig. 5, e, f). These results suggest that the decreased motility of $\Delta put A$ is due to its deformed flagella (Fig. 5, e, f).

Motility and chemotaxis are delicate phenomena and seem to be affected by many environmental factors such as metabolic substances, temperature, oxygen and cell density. Motility is considered to impose a physiological cost, and has been estimated to be about 2% of the total energy expenditure for an enteric bacterial species (12). Proline plays a central role in energy metabolism and can serve as an important energy source for various kinds of enteric bacteria (2). *H. pylori* ATCC 43504 wild strain consumed L-proline rapidly in the Ham's F-12 medium (Fig. 2). Cells of wild-type *H. pylori* cultured in Brucella broth contained small amounts of proline (Fig. 3). Since the amounts of glutamate which is a product of proline respiration were significantly higher in the wild-type than in $\Delta put A$ (Fig. 3), proline seems to be used as the main respiratory substrate in *H. pylori*. Thus, the lack of proline respiration by $\Delta put A$ might be another reason for the decreased motility of this mutant.

In the *H. pylori* 26695 strain, *put A* and *put P* are coded at HP0056 (3558 bp) and HP0055 (1491 bp), respectively. *Put P* is located downstream in the same direction from the promoter region of *put A*, and 15 bp at the beginning of *put P* overlaps with 15 bp at the end of *put A*. Our present work has shown that *put P* encoding L-proline permease did not seem to be transcribed since the disruption of *put A* caused loss of the ability to incorporate L-proline into cells (Fig. 2). These results demonstrated that *put A* and *put P* are co-regulated genetically as one operon.

Put A consisting of two domains, those of proline dehydrogenase and P5C dehydrogenase, is a relatively large polypeptide enzyme containing 1,185 amino acids in *E. coli* (35). As *Put A* and *Put P* have been reported to be membrane-bound in gram-negative bacteria (1, 3), those of *H. pylori* are also likely to be membrane-bound. Thus, the loss of *Put A* together with *Put P* may affect other protein expressions participating in the motility of *H. pylori*, and result in morphological changes of the flagella. Further study, *e.g.* using proteome analysis, is needed to clarify which protein(s) other than *Put A* or *Put P* is (are) affected by the disruption of *put A*.

Kavermann *et al.* reported that *put P* was one of essential genes for gastric colonization in an animal model infected with *H. pylori* (14). Our present works has shown that $\Delta put A$ failed to show colonization in the infected mice (Fig. 6), although there is a possibility that faster clearance of $\Delta put A$ than the wild-type appeared in the infected mice. Thus, it may be possible to develop the $\Delta put A$ of *H. pylori* as a useful vaccine.

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